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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): Huston, James S. et al. EXAMINER: J. Ulm

SERIAL NO.: 07/661,070

GROUP NO.: 185

FILED: February 26, 1991

TITLE: PRODUCT AND PROCESS FOR THE PRODUCTION, ISOLATION AND
PURIFICATION OF RECOMBINANT POLYPEPTIDES

Honorable Commissioner of Patents
& Trademarks
Washington, D.C. 20231

DECLARATION OF DAVID C. RUEGER

UNDER 35 U.S.C. RULE 1.131

I declare:

1. I, David C. Rueger, hold a Ph.D. in Biochemistry from Duke University. I am a named inventor of the above-captioned patent application and hold the position of Senior Research Director at Creative Biomolecules, Inc., the assignee of the above-captioned patent application.

2. Prior to March 28, 1986, I and my co-inventors reduced to practice in the United States the construction and expression of recombinant DNA encoding a fusion polypeptide containing a leader/hinge region, a cleavage site, and a target polypeptide, and the subsequent cleavage of that polypeptide at the cleavage site by a cleavage site-specific protease. The following experiments, performed by Zita Babickas under my direction, demonstrate that a fusion polypeptide having the structure described in the above-captioned patent application is cleaved at the cleavage site nearest the hinge region. These experiments are described in the above-captioned patent application (see Example III, in particular) and in the accompanying Exhibits A and B. The results are shown in Exhibits C-E. These experiments involve fusion polypeptides in which the target protein is calcitonin.

3. Polypeptide 1, described on pages 39-41 of the above-captioned patent application, includes a TRP-LR leader sequence connected at its carboxyl end to a Phe-Pro-Gly hinge, followed by a glutamic acid residue and human calcitonin. As stated in the specification on page 40, lines 1-7, it was desired that the tripeptide hinge would promote cleavage at the Glu residue by providing a flexible residue (proline) and an uncharged "spacer" residue next to the Glu residue. The large leader was used to promote high expression levels. The sequence of polypeptide 1 is provided in the specification on pages 40-41.

4. Polypeptide 2, described on pages 40-41 of the above-captioned patent application, is a smaller polypeptide in which the TRP-LE leader is truncated, a dipeptide Asp-Leu hinge, a Glu cleavage site, and salmon calcitonin analog as a target polypeptide. The salmon calcitonin analog was used because it contained an Asp at position 15 instead of the cleavable native Glu. The sequence of polypeptide 2 is set forth on page 41 of the specification. As stated in the specification on page 41, polypeptide 2 was designed with the goal of obtaining a fusion polypeptide which was cleavable into only 2 fragments, the hook/hinge fragment and calcitonin. Also, the hinge of polypeptide 2 was designed to facilitate cleavage with V-8 protease such that cleavage approached 100% completion.

5. Exhibits A and B, attached hereto, demonstrate the experiments in which polypeptides 1 and 2, respectively, were cleaved with V-8 protease. These experiments were performed and the pages submitted as Exhibits A-E are dated prior to March 28, 1986. Exhibits C, D, and E show the results of these cleavage experiments. Exhibit C shows cleavage of polypeptide 1, in which V-8 protease cleaved the fusion protein into multiple fragments. Exhibit D shows cleavage of polypeptide 2, in which V-8 protease cleaved the fusion protein into the two expected fragments, identified as "hook" (containing the leader and hinge regions) and "SC" (containing the salmon calcitonin analog). Exhibit E

shows the purified salmon calcitonin analog collected from the "SC" peak shown in Exhibit D.

6. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1011 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

David C. Rueger
David C. Rueger

3-18-92
Date

189kam2054/6.AH6

PROJECT Calcitonin 1

Staph cleavages (bulk) of LF-Glu-HC in 4M + 6M urea w/w/o DT

2 ml of C133-pool were dialized overnight up to Murea AcE, pH:
Concentration assumed c. 7 mg/ml as before

| code | μl prot | μl buff | mg prot | ratio | μl enzyme |
|-------|---------|---------|---------|-------|-----------|
| 133-A | 500 | - | .355 | 1:50 | 6.7 |
| -B* | 330 | 170 | .234 | 1:50 | 4.7 |

another shot of 1:50 enzyme added after 3 hr. (4:30 pm) (C133-A, B-2hr+1hr)

Gel samples taken at 2 hr., 2 hr + 3 hr., 3 hr + 28 hr.

12/84 Another shot of 1:50 enzyme added after 2 hrs. (1:30 pm 9/12) (C133-A, B-2hr+1hr)
4M of staph added to AcE 5:30 pm (C133-A-2hr+24hr) + 6 + 18 hr.

| code | μl prot | μl buff | μl DTT | mg prot | ratio | μl enzyme |
|------|---------|---------|--------|---------|-------|-----------|
| C-C* | 200 | 100 | - | 0.142 | 1:50 | 2.84 |
| -D* | 200 | 100 | 10 | 0.142 | 1:50 | 2.84 |

protein incubated w/DTT for 2 hrs before enzyme addition - 2mM DTT final conc
extra shot of 1:50 added after 3 hrs.

C received 2nd extra shot after 3 hrs. (C133-C-2hr+3hr+1hr) ≠
D " " " . . . 18 hrs (C133-D-2hr+18hr+1hr)

13/84

| code | μl prot | μl buff | μl DTT | mg prot | ratio | μl enzyme |
|------|---------|---------|--------|---------|-------|-----------|
| C-F* | 1.0 | .5 | .7 | 1.50 | 13.4 | |

extra shots after 2hr + 18 hrs (C133-F-2hr+18hr+1hr)

pH was ~7.1 after 18hr - brought up to pH 7.9 before addition of enzyme

all 4M urea samples were turbid after ~1hr

Yield: 280 μl collection (94.6 μg injected × 1.4 = 133.2 μg calc expected)

peak weight = 7.5% of total = 7.1 μg = 53.3%

$$\text{OD} = 0.37 \div 0.035 \mu\text{g} = 10.6 \mu\text{g} = 80.3\%$$

Continued on Page

Read and Understood By

C.H.

Jita A. Babickas

PROJECT: Calcitonin 180

Bulk Staph cleavage of reduced SyntrpSC-asp

2 μ l of conc C178-pool made 20w18IV + left at RT for 2 hrs
 Digestion overnight vs 1L 6Murea 20w1NHyAcet E, pH 7.8 (1.3mg/ml)

4/17/85

- (A) 100 μ l of protein (caesine mg/ml) + 50 μ l 20w1NHyAcet E, pH 7.8
 + 1.3 μ l staph (Pierce .64mg/ml) 1:50 excess @ 9:50 (1:70 ratio)
 collected @ 4.5' + 1/2 hrs
- (B) 200 μ l of protein (.26 mg) + 100 μ l 20w1NHyAcet E, pH 7.8
 + 2.7 μ l staph (Pierce .64mg/ml) 1:150 excess @ 12:10
 collected @ 30', 1 hr + 1/2 hrs
- (C) 200 μ l of protein (.26 mg) + 100 μ l 20w1NHyAcet E, pH 7.8
 + 2.7 μ l staph (Pierce .64mg/ml) 1:150 excess @ 2:00
 collected @ 30', 1 hr + 1/2 hrs.
- (D) 200 μ l of protein + 100 μ l 20w1NHyAcet E, pH 7.8
 + 2.7 μ l staph (Pierce .64mg/ml) 1:200 excess @ 4:35
 left at 4°C after ~2 hrs

Yield: ~90 μ g of cSC / collection on LC (100 μ l of each digest)
 ∵ 90 μ g / 100 μ l conc C178-pool
 ∵ 9.5 mg cSC / 10gr cells.

~ 1 mg cSC / g. cell

Continued on Page

Read and Understood By

Bte A. Dabicker

C.H.

EXHIBIT C

MAR 16 '92 13:00 CBM

TRP-LE-calmodulin poly peptide 1
10 V8 cleavage of purified fusion P.8

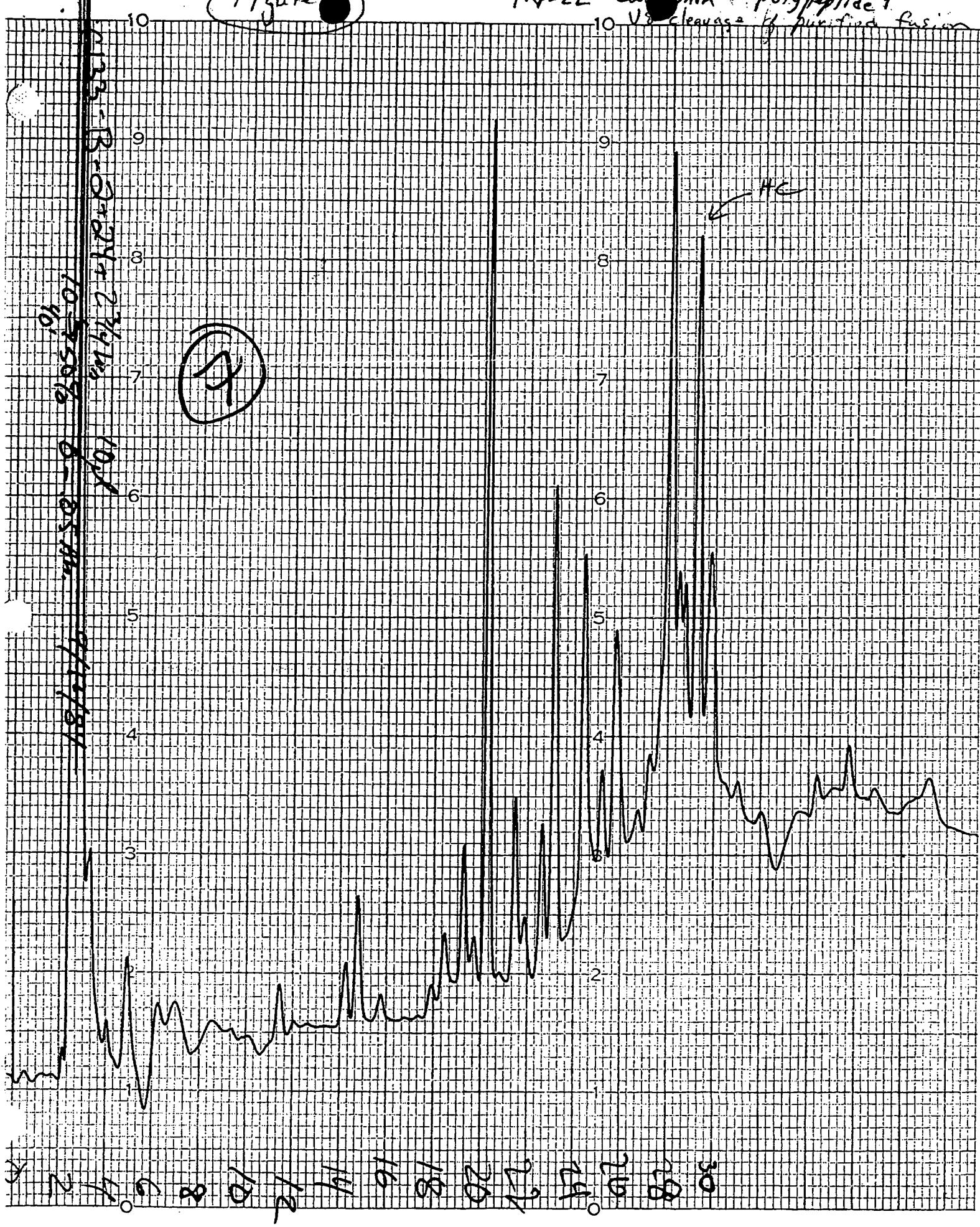
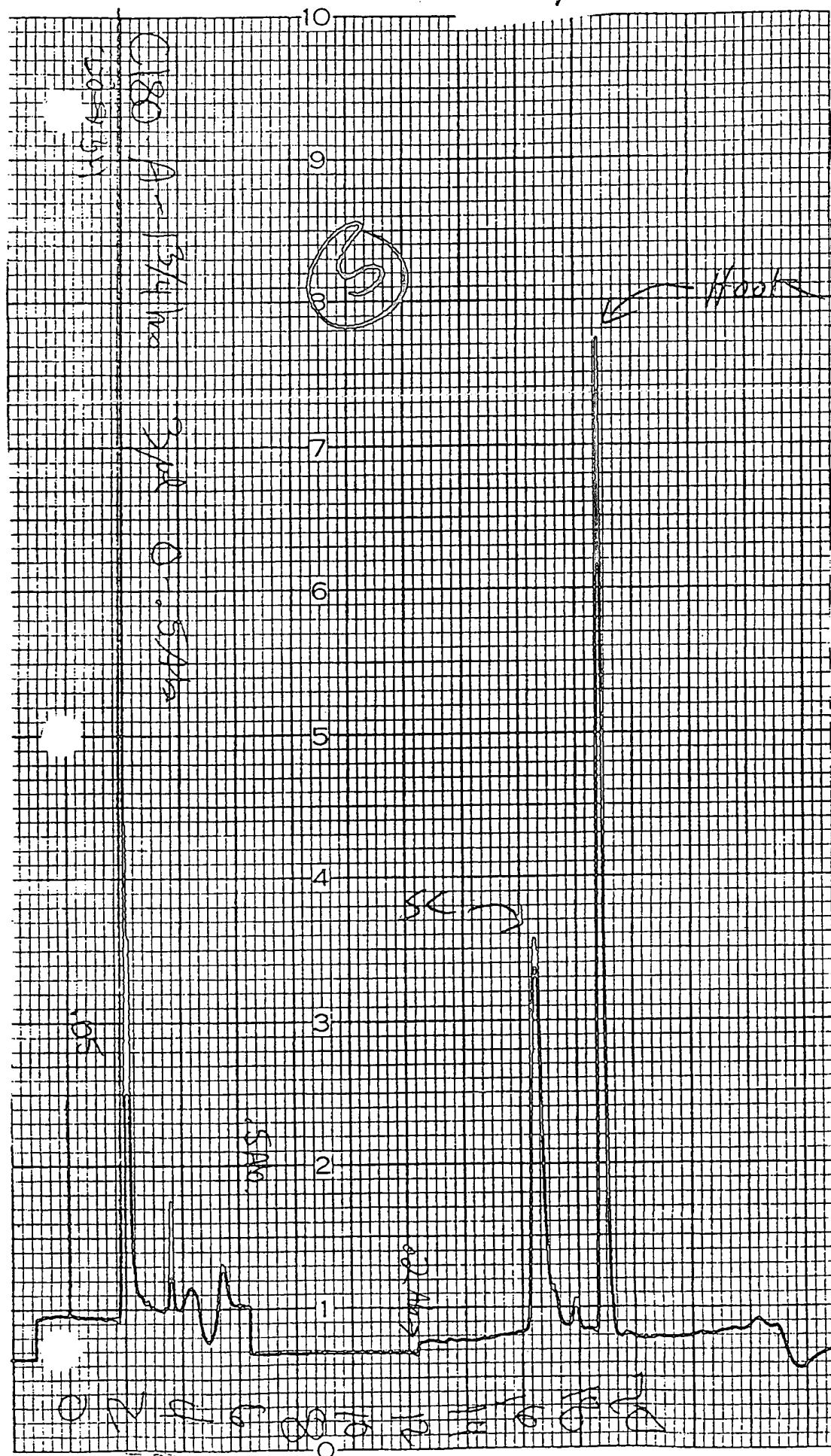


Figure 2

poly peptide 2
V8 cleavage of
united fusion



MAR 16 '92 13:05 CBMtorin
poly peptide 2
V8 cleavage
purified calcitonin

EXHIBIT E

Figure 3

P.11

